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14. ABSTRACT

BRCA1 is the major breast cancer susceptibility gene. It forms heterodimers with BARD1. Inactivation of either gene results in identical phenotypes suggesting that these proteins function primarily as a complex. BRCA1 deficiencies are associated with cellular phenotypes consistent with a DNA replication defect. We wished to test the hypothesis that BRCA1/BARD1 function during DNA replication supporting DNA transactions at replication forks. We are using cell-free extracts derived from Xenopus laevis eggs that support: 1. Semi-conservative, cell-cycle regulated DNA replication; 2. Many facets of the DNA damage response. Our key accomplishments were to generate specific antibodies against Xenopus BARD1 and BRCA1. We also demonstrate that the complex assembles to chromatin in a DNA replication-dependent manner. Finally, we show that BRCA1/BARD1 loading to chromatin does not dramatically increases following DNA damage, suggesting that it might be relocalized within chromatin compartments.

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INTRODUCTION

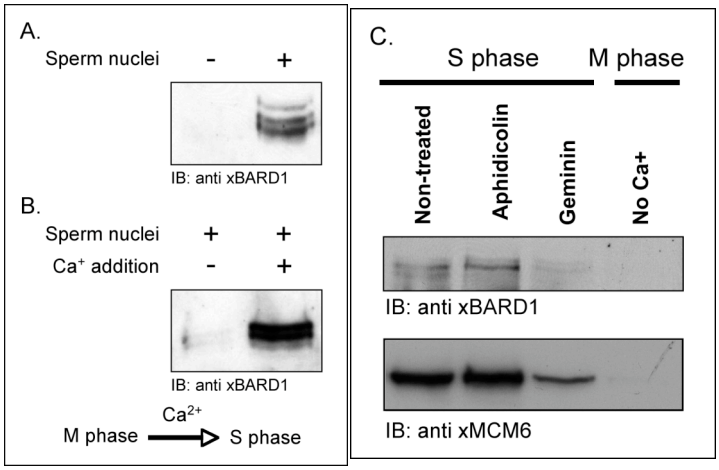
Mutations in the BRCA1 tumor suppressor gene occur in about 40% of familial cases of breast cancer. Carriers of BRCA1 germline mutations have a lifetime risk of 50-85% for breast cancer development. In addition, sporadic breast cancer patients often show reduced BRCA1 expression (Yoshida and Miki, 2004). The BRCA1 protein is expressed in most proliferating cells and was shown to be involved in various cellular processes including DNA repair, transcriptional regulation, chromatin remodeling and cell cycle checkpoint regulation. The BRCA1 protein contains two known amino acid motifs: a RING domain at the N terminus and two tandem copies of the BRCT domain at the C terminus. The BRCA1 RING domain forms a heterodimeric complex with another RING domain protein, BARD1. BARD1 is the major BRCA1 interacting protein in the cell. In knock-out mice, BRCA1 and BARD1 have very similar phenotypes and in Xenopus the two proteins are co-regulaed both at the mRNA and protein level, suggesting that most of BRCA1 functions might require BARD1 (Joukov et al., 2001; McCarthy et al., 2003). Indeed, BRCA1/BARD1 interaction was shown to be required for their proper localization, E3 ligase activity, DNA binding and protein stability. Following DNA damage, the BRCA1/BARD1 complex localizes to nuclear foci along with other proteins that are involved in DNA repair including Rad51 and the Mre11/Rad50/Nbs1 (MRN) complex. Both BRCA1 and BARD1 are phosphorylated in response to exogenous DNA damage. BRCA1 is phosphorylated by ATM in response to DSBs (Cortez et al., 1999) and by ATR in response to replication inhibition (Tibbetts et al., 2000) this may suggest a role for BRCA1/BARD1 complex in DNA repair following replication stress. In addition BRCA1/BARD1 nuclear foci are observed in untreated S-phase cells, supporting a role for BRCA1 and BARD1 during the DNA replication process.

DNA replication is a complex mechanism that requires the coordinated interplay of multiple factors. Many of which are involved in DNA transactions that maintain the integrity of the genome. The replication fork is a structure with the potential to trigger a DNA damage response. DNA replication fork stalling or collapse has been proposed to lead to DSBs formation and was shown to be highly reliant on homologous-recombination. Taken together, it is possible that BRCA1/BARD1 complex exerts its critical role as tumor suppressor at the replication fork where it stabilizes the fork and facilitates DNA transactions triggered by DNA damage. Our objectives are 1) to establish that BRCA1/BARD1 is present at the replication fork; 2) to determine if DNA lesion or fork stalling enhance BRCA1/BARD1 recruitment at the replication fork; 3) to assess the consequences of complete loss of BRCA1/BARD1 on fork progression and stalling.

BODY

Cell-free systems derived from Xenopus eggs can recapitulate cell cycle progression, DNA replication, mitosis, checkpoint response and DNA repair in a test tube (Costanzo et al., 2004). In our lab we have raised antibodies against xBRCA1 and xBARD1. However, only BARD1 antibodies were sensitive enough to detect a 100 kD band in Xenopus extracts as was previously described. As BRCA1/BARD1 is mainly found as a heterodimer in Xenopus extracts we focused on BARD1.

Figure 1. BARD1 associates with chromatin in a replication dependent manner.



In low-speed-supernatant (LSS) extract BARD1 is specifically associated with chromatin (Fig. 1 A). In the absence of Calcium, LSS extracts are arrested at the M phase of the cell cycle. Upon Calcium addition, that mimics fertilization, DNA templates can undergo cell-cycle regulated, semi-conservative DNA replication (Blow and Laskey, 1986). Under this condition a specific 100 kD corresponding to BARD1 chromatin association can be detected. Moreover, in the presence of Geminin, that binds to Cdt1 and inhibit MCM loading onto the chromatin and replication initiation, BARD1 chromatin association was markedly reduced. When using Aphidicolin, that is a DNA polymerase inhibitor that blocks replication elongation, BARD1 chromatin association was not changed. This may indicate that BARD1 loades onto chromatin during the initiation steps of DNA replication.

In order to verify these results we have used a different extract preparation method term HSS (high speed supernatant) and NPE (nucleoplasmic extract). In this system DNA replication is a two-step reaction, where pre-replication complexes (pre-RC) assembly on the DNA template is physically and temporarily dissociated from origin firing. In the first step, pre-RC assembled in membrane-free cytosol (HSS) and this is followed by the addition of NPE that provides the regulatory activities required for firing including Cdk2/cyclinE and Ccd7/dbf4 (Walter et al., 1998). In HSS alone, no BARD1 chromatin association can be detected. As a loading control we have used xMCM6 antibodies. Upon addition of NPE, Cdc45 is loaded and replication can initiate, under these conditions, BARD1 is associated with the chromatin. This binding is not affected by aphidicolin that blocks the later replication initiation step, but is markedly reduced when using geminin that blocks pre-RC formation. In NPE alone that does not support DNA replication BARD1 chromatin binding could not be detected. Thus, we can conclude that BARD1 associates with chromatin during the replication initiation.

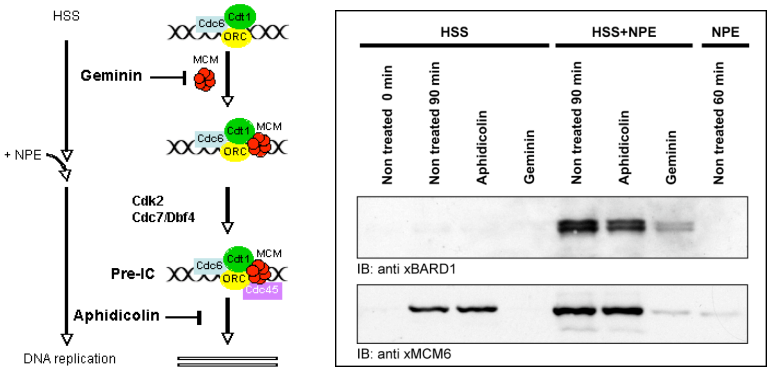


Figure 2. BARD1 loading requires pre-RC assembly. Left panel. Schematic of pre-RC assembly showing the steps that are inhibited by geminin and by aphidicolin.
Right panel. Chromatin loading of BARD1 following the inhibition of pre-RC assembly (Geminin) or DNA replication elongation (aphidicolin).

In response to DNA damage, BRCA1/BARD1 localizes to nuclear foci containing other DNA repair proteins. Next we looked at BARD1 chromatin association in response to different DNA damaging agents. In response to Mitomycin C (150mM) DNA cross linking agent, BARD1 chromatin association did not change. However, and Cisplatin (25mM) which is also a DNA cross-linking agent completely abolished BARD1 chromatin association. This is probably due to high dose as indicated by the lack of MCM6 control loading that indicate that there was no replication. In response to Etoposide (100mM) treatment that inhibit topoisomerase II and induce DNA breaks and inhibit DNA synthesis BARD1 chromatin association did not change. Finally, Camptothecin (50mM) that inhibit topoisomerase I and increased DNA cleavage also did not change BARD1 chromatin association. Altogether, we can conclude the no increase in BARD1 chromatin association can be detected following DNA damage.

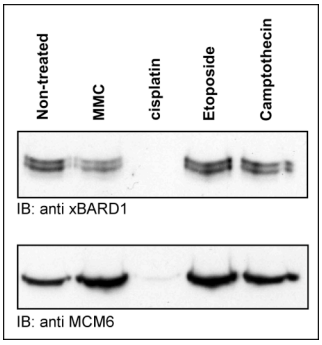


Figure 3: Chromatin-bound BARD1 following DNA damage.
BARD1 loading onto chromatin is not increased following treatments with mitomycin C, a cross-linking agent, etoposide, a DNA topoisomerase II inhibitor or Camptothecin, a DNA topoisomerase I inhibitor

KEY RESEARCH ACCOMPLISHMENTS

- To generate specific antibodies against Xenopus BARD1 and BRCA1.
- To demonstrate that the BRCA1/BARD1complex assembles to chromatin in a DNA replication-dependent manner.
- To show that BRCA1/BARD1 loading to chromatin does not dramatically increases following DNA damage, suggesting that it might be relocalized within chromatin compartments.

REPORTABLE OUTCOME

An abstract will be presented at the Keystone meeting on DNA recombination, January 2006

CONCLUSIONS

We have made significant progress by demonstrating the pre-Replicative-dependent loading of BARD1 onto chromatin. This is a critical accomplishment that is in strong support of our original hypothesis that BRCA1 is part of the replication machinery. It will serve as a base for future efforts. Furthermore, our preliminary data suggests that the BRCA1/BARD1 complex might not be enriched on chromatin following DNA damage but instead be recruited from its replication-dependent localization on chromatin to damaged sites.

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